Determination and Nature of Leaf Sterols

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Methods for the determination of unsaturated and saturated sterols in leaf meals are described. Alternative procedures using micro- or macrosamples and colorimetric or gravimetric techniques are given. The close relationship between the time vs. density curves, absorption curves, and $E\,_{1\,cm.}^{1\%}$ of the color reaction product is demonstrated.

LEAF sterols may have potential value as sources for the preparation of vitamin D-active compounds and sex hormones. In view of that fact it seemed desirable to determine the sterol content of a number of leaf meals and extracts.

No method has been published for the quantitative determination of leaf sterols, although many procedures are available for the determination of cholesterol in blood and animal tissue and sterols in vegetable oils. Sterols in such materials can be determined by macro gravimetric methods or micro colorimetric procedures. The former are all based on the work of Windaus (17), who showed that on the addition of digitonin, cholesterol or phytosterols in alcohol solution form a characteristic insoluble digitonide. The sterol digitonide is then filtered and weighed. All the plant sterols isolated to date can be precipitated by digitonin.
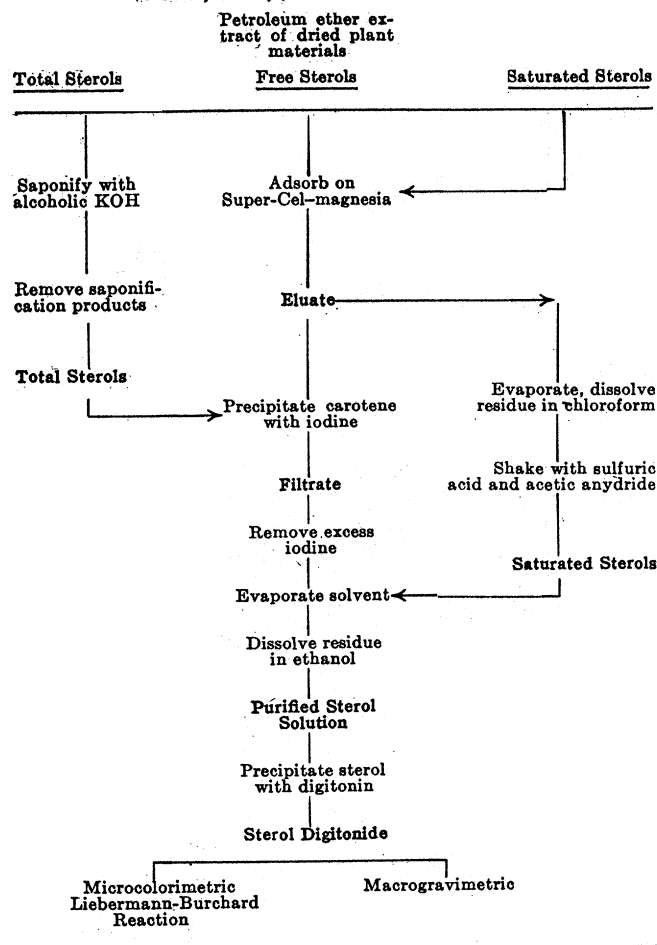
The macroprocedures devised for cholesterol are accurate but have the disadvantages of being time-consuming and requiring large samples and considerable quantities of expensive digitonin. Microprocedures devised for cholesterol eliminate these difficulties. Of these, the Liebermann-Burchard reaction, in which the sterol is treated with acetic anhydride and sulfuric acid, has been the most extensively studied.

Bloor (2) adapted the reaction to determination of cholesterol in blood and many workers have introduced modifications. Schoenheimer and Sperry (12) precipitated cholesterol as the digitonide and, after dissolving the compound in acetic acid, carried out the colorimetric reaction in the presence of digitonin. Kelsey (7) also precipitated cholesterol as the digitonide, which he then decomposed with boiling benzene and, after removing the insoluble digitonin, carried out the color reaction on the free sterol. Sperry (13) has shown that determination of cholesterol by the Schoenheimer-Sperry microprocedure has an accuracy comparable with that of macromethods.

By utilizing a number of the foregoing procedures and intro-

Table I. Essential Steps Involved in Determination of Total, Free, and Saturated Sterols

Petroleum ether extract of dried plant materials

Total Sterols	Free Sterols	Saturated Sterols
Saponify with alcoholic KOH	Adsorb on Super-Cel–magnesia	
Remove saponification products	Eluate	
Total Sterols	Precipitate carotene with iodine	Evaporate, dissolve residue in chloroform
	Filtrate	Shake with sulfuric acid and acetic anydride
	Remove excess iodine	Saturated Sterols
	Evaporate solvent	
	Dissolve residue in ethanol	
	Purified Sterol Solution	
	Precipitate sterol with digitonin	
	Sterol Digitonide	
	Microcolorimetric Liebermann-Burchard Reaction Macrogravimetric	

ducing some necessary purification steps, the authors have determined the sterol content of leaf meals by both macro- and microtechniques.

EXTRACTION OF STEROLS

Dehydrated leaf meals (4 to 8% residual moisture) are ground 30- to 40-mesh, and 25 grams are exhaustively extracted with Skellysolve B in an all-glass Soxhlet apparatus. After cooling, the extracts are transferred to a 100-ml. volumetric flask and made to volume.

DETERMINATION OF FREE STEROLS

Since even minor details are important, the methods are presented as completely as possible. Table I outlines the essential steps in the various procedures.

Microprocedure. PURIFICATION OF SAMPLE. An adsorption column of 7 × 2 cm., filled with a mixture of three parts of Hyflo Super-Cel (Johns-Manville Corporation) and one part of activated magnesia No. 2641 (Westvaco Chloride Products Company) is used (15, 16). A 5- to 10-ml. aliquot of the extract is adsorbed, and the sterols as well as carotene are eluted with 50 ml. of 5% acetone in Skellysolve B followed by 50 ml. of 10% acetone in Skellysolve B. Xanthophyll and chlorophyll are retained on the column. The extract is then evaporated to 5 to 10 ml. on a hot-water bath. All evaporations are conducted in a well-ventilated hood.

The carotene in the concentrated extract is now precipitated as the insoluble iodide (18) by adding 5 ml. of a 0.2% iodine solution in Skellysolve B (prepared by dissolving 200 mg. of finely ground iodine in a few milliliters of ethanol and making to 100 ml. with Skellysolve B). The mixture is kept at −20° C. for 30 minutes to 1 hour.

The carotene iodide precipitate is filtered on a thin layer of

Super-Cel in a small sintered Hirsch funnel and washed thorough with previously cooled Skellysolve B. To remove excess iod the filtrate containing the sterols is transferred to a separat funnel and shaken vigorously with 5 ml. of aqueous 10% sod thiosulfate solution. The thiosulfate layer is drawn off, and extract is shaken several times with 10 to 20 ml. of wat finally with 10 to 20 ml. of 90% methanol. The solution, w should be colorless or light yellow, is run into an Erlenme flask, evaporated on a water bath to approximately 10 ml., made to 25 ml. with Skellysolve B.

PRECIPITATION AND COLORIMETRIC ESTIMATION OF FREE STEROL. The following steps are essentially a combination of the procedures of Kelsey (7) and of Schoenheimer and Sperry (12) and are shorter and simpler than either of the originals:

A 5- or 10-ml. aliquot of the purified extract is pipetted into a 15-ml. conical centrifuge tube. A boiling rod similar to that described by Kelsey is inserted, and the solution is gently evaporated to dryness by heating the centrifuge tube in a water bath containing approximately a 3-cm. depth of water. Without removing the boiling rod 4 ml. of a 0.2% digitonin solution in 95% ethanol (digitonin Merck) are added to the residue. The solution is stirred with the boiling rod and evaporated to dryness. The last traces of alcohol are removed by a gentle current of air. The residue, consisting of sterol digitonides and excess digitonin, is stirred and washed with 7 or 8 ml. of Skellysolve B. The boiling rod with any adhering precipitate is carefully removed and placed on a suitable rack, and the tube is then centrifuged at 2500 to 3000 revolutions per minute for 10 minutes. The petroleum ether is decanted and discarded, and the process is repeated. Finally the last traces of petroleum ether are removed by heating and suction.

The sample is now ready for colorimetric estimation, for which a slight modification of the procedure of Schoenheimer and Sperry is used. Two milliliters of glacial acetic acid are added, and the precipitate is dissolved by heating it gently on a water bath while stirring with the boiling rod. The centrifuge tube is then placed in a water bath at 24° C., and 4 ml. of acetic anhydride are added with stirring with the boiling rod, followed by addition of 0.2 ml. of concentrated sulfuric acid, and further stirring. Thirty minutes after the addition of the acid, the solution is transferred to a micro colorimeter tube, and the density is observed in a photoelectric colorimeter at approximately 6 mμ. A Fisher Electrophotometer provided with one No. 24 Corning red and one No. 4308 Corning light shade blue-green filter was used to give the desired wave length. For the zero setting of the instrument a blank is prepared by evaporating 4 ml. of 0.2% digitonin solution to dryness and then treating in the same manner as the samples.

Since leaf sterols usually occur in mixtures, the composition of which may vary from species to species, and since different sterols may give different types of reactions with the Liebermann-Burchard reagents, no one sterol can be used for standardization purposes. Instead, the sterol digitonide of each leaf species investigated was isolated (see macromethods) and used. Forty milligrams of the dry sterol digitonide were dissolved in 25 ml. of hot glacial acetic acid; the solution thus contained the equivalent of 0.4 mg. of sterol per milliliter. Aliquots containing 0.2, 0.4, 0.6, and 0.8 mg. of sterol were pipetted into centrifuge tubes, and the colorimetric determination was carried out as described previously. In this manner a calibration curve was prepared for the sterols of each type of leaf meal studied.

Macroprocedure. PURIFICATION OF SAMPLE. A 15 × 2 cm. adsorption column containing the previously described Super-Cel-magnesia mixture is prepared. An aliquot of plant extract equivalent to 5 to 10 grams of leaf meal is adsorbed and eluted as in the micromethod.

The solution is then concentrated to 5 to 10 ml. on a hot water bath. At this stage it may be necessary to remove carotene. Although almost identical gravimetric results are obtained whether or not carotene is removed, the sterol digitonide oft has a yellow color when carotene is not eliminated, and therefo iodine precipitation is used when standards for the microdet mination are desired. In such instances, 50 ml. of petrole ether containing 100 mg. of iodine are added to the concentrat extract, and the solution is kept at −20° C. for 30 minutes to 1 hour and then filtered and transferred to a separatory funnel as described under micromethods. Excess iodine is removed b shaking the solution with 50 ml. of 10% aqueous sodium th sulfate followed by several water washes and a final washing wit 90% methanol. The solution is then concentrated to 5 to 10 ml From this point the procedure is the same whether or not caro tene is removed. The concentrated solution is filtered through a thin layer of Super-Cel on a small Hirsch funnel, washed well with Skellysolve B, and transferred to a 150-ml. beaker. The

...ition is evaporated to dryness on a hot water bath and taken [in] 25 ml. of absolute ethanol.

GRAVIMETRIC ESTIMATION. The alcohol solution is brought to [b]oil, and at least a 50% excess of 1% digitonin in 80% alcohol [a]dded. From 5- to 10-ml. of the digitonin solution are suffi[cient] depending on the sample. The rest of the procedure is [iden]tical with method F, described by Sperry (13).

The gravimetric factor 0.253, used to calculate the quantity of sterol from the weight of the digitonide, was obtained by assuming the average empirical formula of the leaf sterols to be $C_{29}H_{48}O$, since all the leaf sterols isolated to date have the composition $C_{29}H_{48}O$ or $C_{29}H_{50}O$. The formula of Merck's digitonin, taken from the Merck Index (9), is $C_{55}H_{90}O_{29}$. One mole each of sterol and digitonin is involved.

DETERMINATION OF TOTAL STEROLS (FREE PLUS COMBINED)

Microprocedure. In order to liberate combined sterols, a 5- to 10-ml. aliquot of the extract is refluxed for 30 minutes with 5 ml. of 10% potassium hydroxide in 95% ethanol. The solution is transferred to a small separatory funnel, Skellysolve B and alcohol being used alternately. Sufficient water is added to cause separation of the Skellysolve B and alcohol layers. The latter is removed and extracted three times with Skellysolve B, and washings are combined with the original petroleum ether solution. The combined solution is then extracted 4 or 5 times with 90% methanol to remove xanthophyll, traces of chlorophyll, and alkali. It is then concentrated to 5 to 10 ml. on a hot-water bath. The carotene is removed, and the sterols are estimated colorimetrically as described above. Since chlorophyll has been removed, the adsorption step is unnecessary in this procedure. Combined sterols are found by deducting the result for free sterols from that for total sterols.

Macroprocedure. An aliquot of extract equivalent to 5.0 to 10.0 grams of leaf meal is refluxed for 30 minutes with 50 ml. of 10% potassium hydroxide in 95% ethanol. The saponification products are removed as in the microprocedure, and after being washed with 90% methanol, the petroleum ether solution is concentrated to 5 to 10 ml. The remaining steps are the same as those in the macromethod. The adsorption step is unnecessary.

Table II. Recovery of Sterols by Digitonin Precipitation Microprocedure

| Sterol | Before Precipitation | | After Precipitation | | Recovery |
	Taken Mg.	Density	Density	Found Mg.	%
Cholesterol	0.20	0.13	0.12	...	90
	0.40	0.27	0.27	0.40	100
	0.60	0.41	0.40	0.59	98.5
	0.80	0.52	0.50	0.76	95
β-Sitosterol	0.20	0.08	0.08	0.20	100
	0.40	0.18	0.18	0.40	100
	0.60	0.27	0.27	0.60	100
	0.80	0.36	0.36	0.80	100
Spinasterol	0.20	0.28	0.28	0.20	100
	0.40	0.56	0.57	0.41	102
	0.60	0.85	0.86	0.61	101

DETERMINATION OF SATURATED STEROLS

This procedure is based on the work of Anderson and Nabenhauer (1), who showed that when a mixture of unsaturated and saturated seed sterols in chloroform is treated with acetic anhydride and concentrated sulfuric acid, the unsaturated sterols [pa]ss into the acid layer and the saturated stay in the chloroform.

An aliquot equivalent to 5.0 to 10.0 grams of leaf meal is ad[sorb]ed and eluted as described and then evaporated to dryness. [Th]e residue is taken up in 50 ml. of chloroform and transferred [in]to a glass-stoppered bottle. The bottle is immersed in cold water, and 5 ml. of acetic anhydride are added, followed by the cautious addition of a total of 5 ml. of concentrated sulfuric acid. The [con]tents of the bottle are carefully mixed and cooled continu[ous]ly to room temperature. Then the stopper is tightly inserted, [a]nd the bottle is vigorously shaken for 5 minutes on a shaking [m]achine. The chloroform is decanted from the acid layer into [a] separatory funnel, and the acid layer in the bottle is washed several times with chloroform, the chloroform being decanted each time. The chloroform solution is washed several times with 50% aqueous alcohol to remove traces of acid, and is then

evaporated to dryness. The residue is taken up in 25 ml. of ethanol, and the sterols are precipitated and weighed as usual.

VALIDITY OF PROCEDURE

The fundamental steps involved in these methods are precipitation of sterols with digitonin, removal of chlorophyll and xanthophyll by adsorption, and precipitation of carotene with iodine. As pointed out below, little or no loss of sterol occurs as a result of these techniques.

Digitonin Precipitation. Although digitonin has been used to precipitate animal sterols for many years, it has not been used extensively with plant sterols and, so far as the authors know, never with leaf sterols. It was necessary, therefore, to prove that the reaction was quantitative for leaf sterols.

The microprocedure was tested with solutions of pure cholesterol, β-sitosterol from buckwheat leaves, and spinasterol from spinach leaves. Aliquots of the same solutions were analyzed colorimetrically, without the digitonin precipitation. The results, presented in Table II, show that little loss of sterol occurs in the precipitation step. With plant extracts it is necessary to prepare the sterol digitonide, because even after the preliminary purification steps some extraneous lipides remain and cause high results.

Determinations of cholesterol by the macromethod resulted in 98 to 100% recovery. The gravimetric factor used, 0.242, was based on the molecular weights of cholesterol and of digitonin. A 40.0-mg. sample of the cholesterol digitonide thus secured was dissolved in 25 ml. of warm glacial acetic acid. Aliquots containing the equivalent of 0.2, 0.4, 0.6, and 0.8 mg. of cholesterol were used for colorimetric determinations. The results were practically identical with those shown for cholesterol in Table II, indicating that the cholesterol digitonide was pure.

By using the factor 0.253, this method gave 96 to 98% recovery in the case of spinasterol. When this digitonide was used for colorimetric analysis, results similar to those in Table II were obtained.

The colorimetric procedure is standardized by means of the digitonides isolated by the gravimetric method. If these digitonides were contaminated with other lipides or excess digitonin, or if the precipitation of the sterols were incomplete, the two methods could not give the same results except by a fortuitous combination of positive and negative errors. With the exception of a few cases in which saturated sterols were present, results by micro- and macromethods were in excellent agreement. Since the final determination is based on fundamentally different principles, colorimetric in one case and gravimetric in the other, the fact that the two methods give concordant results (see Table VIII) is good evidence of the validity of the procedures.

Effect of Adsorption. In both procedures chlorophyll must be removed before the digitonin precipitation, since it adheres tenaciously to the precipitate, absorbs light strongly at 620 $m\mu$, and, in the gravimetric method, would be weighed along with the sterol digitonides. If free sterols alone are being determined, the chlorophyll cannot be removed by saponification, for this would also liberate any combined sterols present and therefore adsorption is necessary.

The effect of adsorption on recovery of pure sterols is shown in Table III. The sterols used were β-sitosterol (isolated from buckwheat leaves, melting point 138–139° C.; sitosterol acetate, melting point 128–129° C.) and spinasterol prepared from spinach leaves by the method of Fernholz and Moore (3) (melting point 167–168° C., spinasterol acetate, 180–181° C., benzoate, 196–198° C.). The evidence presented below indicates that most of the leaf sterols investigated are identical with or closely related to these sterols.

The samples were adsorbed and eluted from the magnesia column in the described manner, and the quantity of sterol was determined by the colorimetric procedure. As shown in Table III, both β-sitosterol (paired experiments 1-2-3) and spinasterol

Table III. Effect of Adsorption and Iodine Treatment on Pure Leaf Sterols

Sterol	Expt. No.	Treatment Adsorption	Treatment Iodine	Sterol Taken Mg.	Sterol Found Mg.
β-Sitosterol	1	+	−	0.40	0.39
	2	+	−	0.80	0.78
	3	+	−	0.80	0.76
β-Sitosterol plus 1.0 mg. of carotene	4	−	+	0.80	0.78
	5	−	+	0.80	0.77
Spinasterol	6	+	−	0.20	0.22
	7	+	−	0.40	0.39
	8	−	+	0.20	0.19
	9	−	+	0.40	0.39
	10	+	+	0.20	0.21
	11	+	+	0.40	0.39
Spinasterol plus spinach extract	12	+	+	2.80	2.75
	13	+	+	2.80	2.76

(paired experiments 6-7, 10-11, 12-13) could be adsorbed and eluted from the Super-Cel–magnesia columns with a maximum loss of 5%.
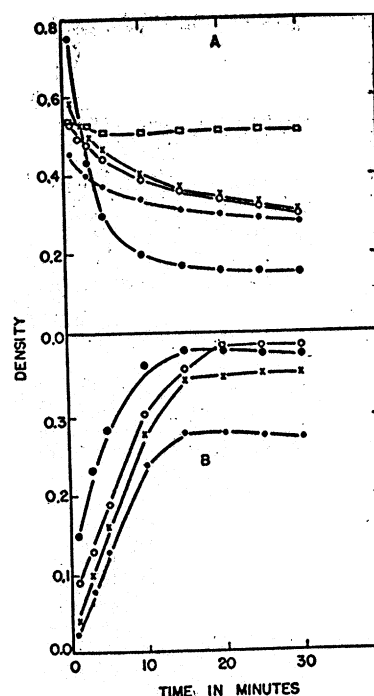
Since the sterols in various leaf extracts might not behave in the same manner as the pure sterols, the adsorption technique was tested with a variety of extracts. In a test with spinach and lima bean leaf extracts, one series was saponified but not adsorbed, the second was saponified and then adsorbed, and the third was adsorbed and then saponified. If there was no loss of sterol due to the adsorption treatment, all three treatments should give comparable results. Table IV (experiments 14, 15, and 16 and 22, 23, and 24) shows this to be true. A similar experiment with beet tops (experiments 29 and 30) also shows that there is no loss of sterol due to adsorption.

The foregoing experiments were all made with the colorimetric microtechnique. The macroprocedure, in which a longer adsorption column and more adsorbent are used, was also tested. In one series of experiments samples were analyzed by both the micro- and macroprocedures, and results in close agreement were found (Table IV, experiments 18-19, 25-26, 35-36). In experiments 26, 27, 32, 33, and 34, samples which contained no combined sterols were analyzed. In such cases, if there was no loss of sterol due to adsorption, samples saponified but not adsorbed should yield the same results as samples adsorbed but not saponified. The results were the same.
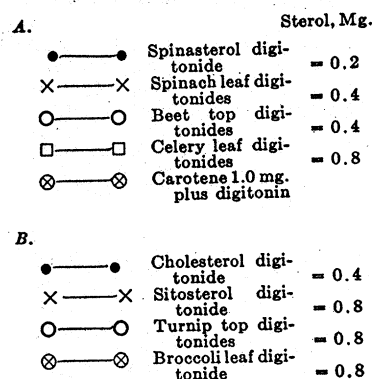
Effect of Carotene and of Iodine Treatment. When leaf extracts containing carotene and sterols are evaporated with the digitonin solution as in the microprocedure, the carotene is tenaciously adsorbed and cannot be completely washed out. Solutions of β-sitosterol and spinasterol to which carotene was added gave colorimetric results much too high. Pure carotene treated in this way gave a fading blue color with the Liebermann-Burchard reagents. Figures 1, A, and 2 show the effect of time on this reaction and the transmission curve of the colored complex formed. From the data it is apparent that carotene must be removed.

Carotene, a highly unsaturated compound, reacts readily with iodine in many solvents to give an insoluble carotene iodide (18), the composition of which varies with the experimental conditions. The effect of this method of separating carotene on the recovery of the sterols was checked in several ways.

Samples of β-sitosterol and spinasterol were treated with iodine and carried through the microprocedure. The results shown in Table III (experiments 4-5, 8-9,

Figure 1. Effect of Time on Rate of Color Development in Liebermann-Burchard Reaction

A.   Sterol, Mg.

●——● Spinasterol digitonide = 0.2
X——X Spinach leaf digitonides = 0.4
O——O Beet top digitonides = 0.4
□——□ Celery leaf digitonides = 0.8
⊗——⊗ Carotene 1.0 mg. plus digitonin

B.

●——● Cholesterol digitonide = 0.4
X——X Sitosterol digitonide = 0.8
O——O Turnip top digitonides = 0.8
⊗——⊗ Broccoli leaf digitonide = 0.8

Table IV. Effect of Adsorption, Iodine, and Saponification on Sterols in Leaf Meal Extracts

Leaf Meal	Expt. No.	Method	Adsorbed	Iodine	Saponified	Free Sterol Mg./g.	Total Sterol Mg./g.
Spinach	14	Micro	−	+	+	...	1.75
	15	Micro, saponification-adsorption	+	+	+	...	1.80
	16	Micro, adsorption-saponification	+	+	+	...	1.82
	17	Micro, saponification 4 hours	−	+	+	...	1.75
	18	Micro	+	+	−	1.53	...
	19	Macro	+	+	−	1.46	...
	20	Micro	−	+	+	...	1.8
	21	Macro	−	+	+	...	1.
Lima bean	22	Micro	+	+	−	...	1
	23	Micro, saponification-adsorption	+	+	−	...	1
	24	Micro, adsorption-saponification	+	+	+	...	1.
Alfalfa	25	Micro	+	−	−	0.80	...
	26	Macro	+	−	−	0.78	...
	27	Macro	−	+	+	...	0.80
	28	Macro	−	+	+	0.94	0.7
Beet top	29	Micro	+	+	−	...	0.9
	30	Micro	−	+	+	...	0.95
Broccoli	31	Macro	−	−	−	3.27	...
	32	Macro	+	−	−	3.28	...
	33	Macro	+	−	+	...	3.15
	34	Macro	−	−	+	...	
Corn	35	Micro	+	+	−	1.03	...
	36	Macro	+	−	−	1.00	...

Table V. Separation of Saturated from Unsaturated Sterols (Free Sterols)

Leaf Meal	1[a] Saturated + Unsaturated Sterols Mg./g.	2[b] Saturated Sterols Direct Mg./g.	1 − 3 Indirect Mg./g.	3[c] Unsaturated Sterols Direct Mg./g.	1 − 2 Indirect Mg./g.
Broccoli	3.22	1.63	1.77	1.45	1.59
Cabbage	0.88	0.53	0.40	0.48	0.35
Kale	4.36	2.90	2.76	1.60	1.46
Pea	2.50	1.40	1.30	1.20	1.10
Spinach	1.46	0.10	−0.07	1.53	1.36

[a] Standard gravimetric procedure.
[b] Gravimetric determination after removal of unsaturated sterol.
[c] Colorimetric determination.

Table VI. Precision of Data Obtained by Micro- and Macromethods

Leaf Meal	Weight of Sample[a] Grams	Procedure	Sterols Mg./g.	Deviation from Average %
Beet	0.25	Micro, unsaponified	0.96	+1.1
	0.50		0.94	−1.1
	0.25		0.95	0.0
	0.50		0.94	−1.1
Alfalfa	0.25		0.79	−1.3
	0.50		0.80	0.0
	0.25		0.80	0.0
	0.50		0.80	0.0
Corn	0.25		1.00	0.0
	0.50		1.00	0.0
Spinach	0.25	Micro, saponified	1.85	+1.4
	0.25		1.80	−1.4
Spinach	10.0	Macro, saponified	1.86	+0.5
	10.0		1.84	−0.5
Kale	2.5	Macro, unsaponified	4.30	−0.2
	5.0		4.36	+1.1
	10.0		4.29	−0.5
	10.0		4.30	−0.2
Broccoli	2.5	Macro, unsaponified	3.20	+0.6
	5.0		3.28	+3.0
	10.0		3.18	0.0
	12.5		3.06	−4.0

[a] Actual sample is extract equivalent to various weights indicated.

10-11, 12-13) indicate that under these conditions the iodine treatment does not affect the recovery of these sterols.

Additional proof of this is shown in the following experiments (Table IV). Leaf extracts analyzed by the microprocedure, in which the iodine treatment is necessary, gave results which agreed closely with those of samples analyzed by the macromethod in which no iodine was used (experiments 25-26, 30-31, and 35-36). Other tests compared samples run by the macroprocedure in which iodine was used with those analyzed by the same method but without iodine (experiments 27-28 and 32-33). Again the results were in excellent agreement. It is apparent, therefore, that the analysis of both pure sterols and sterols in leaf extracts is not affected by the iodine precipitation of carotene.

Effect of Saponification. The effect of saponification on sterols was not exhaustively studied, and the data are somewhat indirect. Certain of the leaf meals, such as alfalfa and beet top, apparently do not contain combined sterols. In both cases, results obtained on unsaponified and saponified samples agree closely in both the micro- and macroprocedures (Table IV, experiments 25, 26, 27, 28 and 29, 30, 31), indicating that the sterols are not affected by the saponification treatment. As shown in experiments 14 and 17, hydrolysis for even longer periods has no effect.

Saturated Sterols. The Liebermann-Burchard reaction is positive only for unsaturated sterols (5), whereas both saturated and unsaturated are precipitated by digitonin and if present in a mixture are estimated together by the gravimetric method. In the case of broccoli, cabbage, kale, and pea vine leaves a great discrepancy was found between the results obtained by the colorimetric and gravimetric procedures. The possibility that this might be due to the presence of saturated sterols was investigated.

The bromination method of Schoenheimer (11) was first tried, but could not be successfully applied to the plant extracts. The procedure of Anderson and Nabenhauer (1) was then tested.

When the sterols in chloroform or carbon tetrachloride solution are shaken with acetic anhydride and concentrated sulfuric acid the unsaturated sterols form a deep blue complex, which remains in the acid layer. The saturated sterols remain in the solvent and can be determined gravimetrically. This procedure gave data which indicate the presence of saturated sterols in the four leaf extracts mentioned previously.

From the data in Table V it is apparent that the removal of unsaturated sterols by this procedure is almost quantitative. Fairly good agreement is found between the direct determination of saturated sterols and indirect results obtained by deducting the values of the colorimetric procedures from those of the corresponding gravimetric methods. With spinach, very little saturated sterol was found, as indicated by the micro and macro data obtained by the conventional procedures. With broccoli, cabbage, kale, and pea vine leaves at least 50% of the sterols in the mixture appeared to be saturated.

Thus there is some evidence for the presence of saturated sterols, but only isolation and establishment of the structure of the sterols in question can definitely prove whether or not they are saturated.

Accuracy and Precision of Colorimetric and Gravimetric Procedures. Since the investigation deals with the determination of a variable mixture of sterols, no data bearing on the intrinsic accuracy of the procedures can be given. It has been shown that the various steps in the procedure do not cause undue loss of sterol and that results by the two methods based on different principles of estimation are in good agreement. Replicate analyses in Table VI indicate that the precision of both the micro- and macromethods is better than ±5%.

Liebermann-Burchard Reaction as Applied to Leaf Sterol Digitonides. TIME-DENSITY RELATIONSHIP. When the Liebermann-Burchard reaction was applied to unsaturated leaf sterols, three characteristic types of time-density relationships shown in Figure 1 were found, as discussed on the following page.
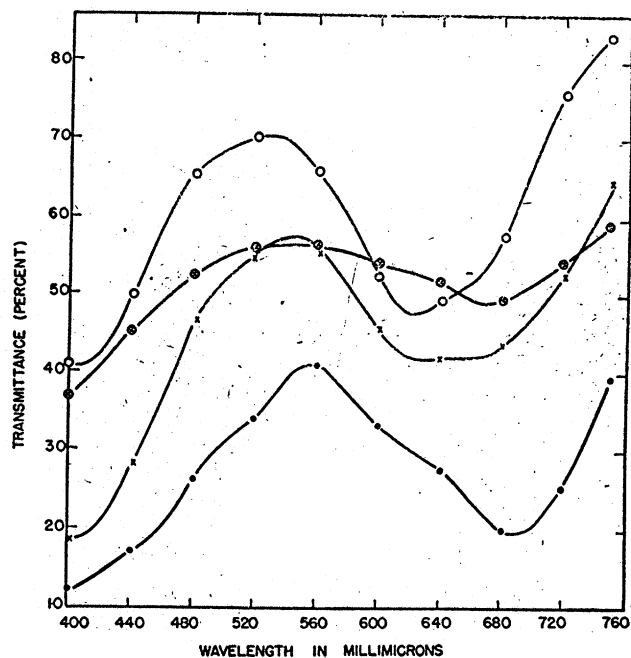

Figure 2. Transmittance Curves of Pure Sterol Digitonides and Carotene after Liebermann-Burchard Reaction

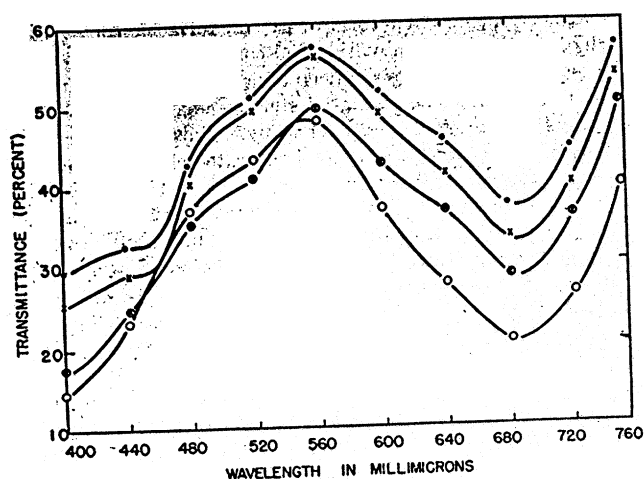|		Sterol, Mg.
●——●	Spinasterol digitionide	= 0.4
○——○	Cholesterol digitonide	= 0.4
×——×	Sitosterol digitonide	= 0.8
⊗——⊗	Carotene 1.0 mg., plus digitonin	

Figure 3. Transmittance Curves of Color Produced by Liebermann-Burchard Reaction with Leaf and Root Sterols Related to Spinasterol

		Sterol, Mg.
•——•	Spinach leaf digitonide	= 0.4
○——○	Celery leaf digitonide	= 0.8
×——×	Beet leaf digitonide	= 0.4
⊗——⊗	Beet root digitonide	= 0.45

1. Maximum color density is attained immediately. The density then decreases, at first rapidly and then more slowly, approaching constancy at 30 minutes. Spinasterol and the sterols from alfalfa, beet, and spinach leaves give this characteristic reaction.

2. The color density reaches a maximum at once and remains constant for 30 minutes. This type of reaction is noted with celery, lima bean, and pea vine leaves.

3. The color density is minimal immediately after the reaction begins, then slowly increases, approaches a maximum in 15 to 20 minutes, and remains constant up to 30 minutes. This type of reaction occurs with cholesterol, sitosterol, and many leaf sterols.

ABSORPTION MAXIMA AND $E_{1\,cm.}^{1\%}$ VALUES. Transmission curves with characteristic absorption maxima were observed with the colored complex formed by the Liebermann-Burchard reaction with leaf sterols. (The authors are greatly indebted to B. A. Brice and M. L. Swain of this laboratory for these data, which were obtained with a G. E. self-recording spectrophotometer.) Typical transmittance curves of the color reaction are shown in Figures 2, 3, and 4.

Two distinct types of transmission curves were found, one having a broad absorption band between 620 and 680 mμ and the other a rather sharp absorption band at 680 mμ. All the leaf sterols tested can be placed in one or the other of these two groups. Spinasterol and the sterols of alfalfa, beet, spinach, celery, lima bean, and pea vine leaves showed the narrow absorption maxima at 680 mμ. β-Sitosterol and many leaf sterols had the wide absorption band from 620 to 680 mμ.

The $E_{1\,cm.}^{1\%}$ values for the Liebermann-Burchard reaction product were calculated from the absorption maxima of the transmission curves 30 minutes after the reaction started.

As shown in Table VII there is a definite association between type of color development, absorption maxima and extinction, in that all sterols in type 1 have an absorption maxima at 680 to 685 mμ and $E_{1\,cm.}^{1\%}$ values of about 75; those of type 2 have the same absorption maximum but $E_{1\,cm.}^{1\%}$ values of about 54; and those in type 3 have an absorption maximum of 620 to 680 and an $E_{1\,cm.}^{1\%}$ value of about 30. The only discrepancies noted were in the case of kale, broccoli, and cabbage, which contain saturated as well as unsaturated sterols. In these cases the $E_{1\,cm.}^{1\%}$

values before correction for saturated sterols were comparable to those of other products in the group, but after correction the values were much higher.

NATURE OF LEAF STEROLS

Although the evidence from the various characteristics of the Liebermann-Burchard-sterol reaction product may be too scanty to cast much light on the nature of the leaf sterols, it may not be amiss to present these facts.

Spinasterol, 3-β-hydroxy-24-ethyl $\Delta^{8(14)22}$ or $\Delta^{8(9)22}$-cholestadiene, has been isolated from spinach and alfalfa leaves (3, 4, 6, 8, 14). The location of the double bond in the phenanthrene ring is considerably different from that of most naturally occurring sterols, and this may be responsible for the difference in the color reaction. The digitonides of spinasterol and of the sterols of alfalfa, beet, and spinach leaves and beet roots all give the same type of color reaction, and hence it is conceivable that the predominant sterol in these products is spinasterol.

The various sitosterols are of widespread occurrence and have usually been found in seed oils. β- and γ-sitosterol (10) are the most common and are isomers differing only in the configuration of the ethyl group on carbon 24, with the structure 3-β-hydroxy-24-ethyl Δ^5 cholestene. The digitonides of β-sitosterol and of the sterols of broccoli, cabbage, carrot, corn, iris, kale, lily, and turnip leaves and of carrot and turnip roots all give similar color reactions, which are different in every respect from the spinasterol group. It seems probable that the sterols of these products are sitosterols, although the evidence is not sufficient to distinguish the various possible sitosterol isomers which may be present.

The sterols of celery, lima bean, and pea leaves give color reactions that cannot be classed with those of either the spinasterol or sitosterol group, although they seem more closely related to the former.

STEROL CONTENT OF LEAF AND ROOT MEALS

The complete data found for various leaf samples are shown in Table VIII. The following facts are apparent. The sterols of most leaves were unsaturated. In cases where saturated sterols were found, they constituted about 50% of the total sterol fraction. In contrast to cholesterol in blood, only a small proportion and sometimes none of the leaf sterols were in a combined form which could be liberated by alkaline hydrolysis. The un-

Table VII. Characteristics of Color Produced by Liebermann-Burchard Reaction with Sterol Digitonides

Source of Sterol	Type of Color	Absorption Maximum mμ	$E_{1\,cm.}^{1\%}$ 30 Min. 24° C. mμ
Spinasterol	I[a]	680–685	110.0, 680
Alfalfa leaf	I[a]	680–685	79.2 680
Beet leaf	I[a]	680–685	76.5 680
Beet root	I[a]	680–685	77.5 680
Spinach leaf	I[a]	680–685	76.5 680
Celery top	II[b]	680–685	54.4 680
Lima bean leaf	II[b]	680–685	54.4 680
Pea leaf	II[b]	680–685	48.0 680[d]
Cholesterol	III[c]	620	50.0, 620
β-Sitosterol	III[c]	620–680	29.2 620
Broccoli leaf	III[c]	620–680	56.0 620[d]
Cabbage leaf	III[c]	620–680	58.0 620[d]
Carrot leaf	III[c]	620–680	33.4 620[d]
Carrot root	III[c]	620–680	29.4 620
Corn leaf	III[c]	620–680	32.0 620
Hemerocallis leaf	III[c]	620–680	31.8 620
Iris leaf	III[c]	620–680	31.0 620
Kale leaf	III[c]	620–680	79.2 620[d]
Lilium canadense leaf	III[c]	620–680	28.0 620
Turnip leaf	III[c]	620–680	29.4 620[d]
Turnip root	III[c]	620–680	31.0 620

[a] Maximum color at start of reaction; then decreases.
[b] Maximum color at start of reaction; remains constant.
[c] Minimum color at start of reaction; then increases.
[d] Corrected for presence of saturated sterols.

Table VIII. Sterol Content of Vegetable Leaf and Root Meals by Micro- and Macromethods

Sample	Total Unsaturated Sterols		Combined Unsaturated Sterols		Free Unsaturated Sterols		Free Saturated Sterols,
	Macro Mg./g.	Micro Mg./g.	Macro Mg./g.	Micro Mu./g.	Macro Mg./g.	Micro Mg./g.	Macro Mg./g.
Alfalfa leaf	0.80	0.79	0.02	0.00	0.78	0.80	...
Beet top	0.95	0.94	0.02	0.00	0.93	0.94	...
Beet root	...	0.25	...	0.00	0.27	0.25	...
Broccoli	...	1.66	...	0.21	1.59	1.45	1.63
Cabbage	...	0.72	...	0.24	0.35	0.48	0.53
Carrot top	...	1.16	...	0.00	0.98	1.16	...
Carrot root	...	0.65	...	0.12	0.55	0.53	...
Celery top	...	1.44	...	0.43	1.12	1.01	....\
Corn leaf	...	1.25	...	0.25	1.00	1.00	...
Iris leaf	0.87	0.94	...	0.27	...	0.67	...
Kale leaf	...	1.74	...	0.14	1.46	1.60	2.90
Lily leaf Canadian	0.71	0.78	...	0.17	...	0.61	...
Hemerocallis leaf	0.81	0.88	...	0.23	...	0.65	...
Lima bean leaf	...	1.44	...	0.43	1.00	0.97	...
Pea leaf	...	1.42	...	0.22	1.10	1.20	1.30
Spinach leaf	1.85	1.83	0.39	0.30	1.46	1.53	...
Turnip top	...	1.60	...	0.44	1.20	1.16	...
Turnip root	...	0.63	...	0.00	0.62	0.63	...

saturated free sterol content of the leaves studied ranged from 0.6 to 1.6 mg. per gram. Corresponding roots contained considerably smaller quantities, ranging from 0.25 to 0.6 mg. per gram.

SUMMARY

Sterols were extracted from leaf meals with petroleum ether and then determined by colorimetric micro- or gravimetric macromethods. In the microprocedure the extract containing sterols was purified by absorption and elution from a Super-Cel–activated magnesia adsorbent column. Carotene, which interferes with the colorimetric determination, was removed by precipitation with iodine. The sterols were precipitated with digitonin according to a combination of the methods of Kelsey and of Schoenheimer and Sperry, and estimated colorimetrically by means of the Liebermann-Burchard reaction with the sterol digitonides.

In the macroprocedure, the sterols were purified in the same manner as in the micromethod, except that carotene was not removed. They were then taken up in alcohol, precipitated with digitonin, filtered on tared crucibles, and weighed. The corresponding data for digitonides obtained by the macromethod were used as colorimetric standards.

In both the micro- and macromethods, combined sterols were liberated by alkaline hydrolysis. After removal of the saponification products, the procedures were similar to the ones described above.

Results obtained by the two methods were generally in good agreement. The precision of both procedures was better than ±5%.

Saturated as well as unsaturated sterols have been found in a few leaf meals. They were separated by the procedure of Anderson and Nabenhauer. The saturated sterols were then estimated gravimetrically.

The characteristics of the Liebermann-Burchard color reaction with sterol digitonides can be used for a general classification of unsaturated leaf sterols. Agreement in rate of color development, absorption maxima, and $E_{1cm.}^{1\%}$ values of spinasterol and β-sitosterol digitonide reaction products with those of the various leaf digitonides, showed that many of the predominant leaf sterols were closely related to or identical with these two sterols.

Free unsaturated sterols occurred in the leaves examined in concentrations from 0.6 to 1.6 mg. per gram. Combined sterols did not occur in appreciable quantities. Free saturated sterols were found in a few leaves, but in those instances the total free sterol content was high, concentrations being as great as 4.0 mg. per gram. In all such cases, the saturated fraction constituted 50% or more of the total sterol.
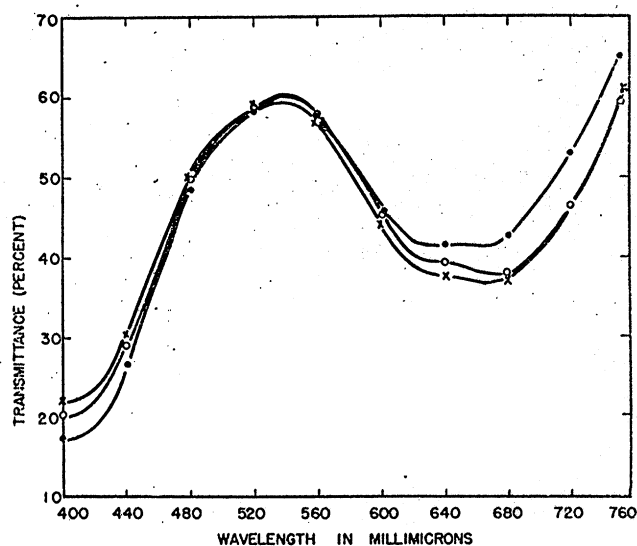

Figure 4. Transmittance Curves of Color Produced by Liebermann-Burchard Reaction with Leaf and Root Sterols Related to Beta-Sitosterol

Sterol, Mg.

●———●	Turnip top digitonide	= 0.8
×———×	Turnip root digitonide	= 0.85
O———O	Corn leaf digitonide	= 0.8

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